Supporting Information

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SI Materials and Methods

Cell-Free Assay for Decaprenylphosphoryl Arabinose Biosynthesis. ***Preparation of the mycobacterial membrane and cell envelope enzymatic fraction.*** About 2 g cell pellet *Mycobacterium smegmatis* mc^2155 grown in LB medium (Invitrogen) supplemented with 0.05% Tween 80 was washed with buffer A (50 mM Mops, pH 7.9, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$) and suspended in 10 mL same buffer. The cells were disintegrated by probe sonication performed in 30-s pulses with 90-s cooling pauses (repeated 20 times). The sonicate was centrifuged at 15,600 × *g* for 20 min at 4 °C, and the resulting pellet was used for preparation of the cell envelope enzymatic fraction as described (1) with minor modifications. Briefly, the pellet was homogenized with buffer A to the volume of 4 mL of the final suspension, to which 6 mL Percoll (GE Healthcare) was added; the mixture was centrifuged at 15,600 × *g* for 60 min at 4 °C. The white upper band was collected, and Percoll was removed from the sample by repeated washings with buffer A and centrifugations at 15,600 × *g* for 20 min at 4 °C. The final pellet was resuspended in 400 μL buffer A, resulting in the sample with a protein concentration of 6.8 mg/mL, which was used as the source of the cell envelope enzyme in the cell-free reactions. Membrane fraction with protein concentration of 49 mg/mL was prepared by centrifugation of 15,600 × *g* supernatant of the sonicate at 100,000 × *g* as described (1).

Composition and analysis of the cell-free reactions. The reaction mixtures contained 75,000 dpm phospho-[^{14}C]-ribose diphosphate (2), 0.1 mM NADH, 3.125% DMSO, 500 μg membrane protein, or 200 μg cell envelope protein and buffer A in the final volume of 80 μL. TCA1 and benzothiazinone 043 (BTZ043) dissolved in DMSO were added to the reaction mixtures in the final concentration of 25 μg/mL. For dose-dependence experiments, TCA1 was added in the final concentrations 1, 3, 6, 12, and 25 μg/mL in the reaction mixtures. After 1 h incubation at 37 °C, the reactions were stopped by the addition of 1.5 mL $CHCl_3/CH_3OH$ (2:1). After 20 min of extraction of the reaction products at room temperature, 170 μL buffer A were added, and the tubes were thoroughly mixed and then briefly centrifuged at 3,000 × *g* to achieve separation of two phases of the mixture (3). The upper aqueous phase containing unreacted radiolabeled substrate was discarded; the bottom organic phase was transferred to the new tube and dried under the stream of N_2 at room temperature. The organic extract was dissolved in 40 μL $CHCl_3/CH_3OH/H_2O$/conc. NH_4OH (65:25:3.6:0.5) and analyzed by TLC on aluminum-backed silica gel plates (F_{254}; Merck) in $CHCl_3/CH_3OH$/1M CH_3COONH_4/conc. NH_4OH/H_2O (180:140:9:9:23). Radiolabeled compounds were visualized by autoradiography (BioMax MR film; Kodak).

1. Mikusová K, et al. (2005) Decaprenylphosphoryl arabinofuranose, the donor of the D-arabinofuranosyl residues of mycobacterial arabinan, is formed via a two-step epimerization of decaprenylphosphoryl ribose. *J Bacteriol* 187(23):8020–8025.
2. Scherman MS, et al. (1996) Polyprenylphosphate-pentoses in mycobacteria are synthesized from 5-phosphoribose pyrophosphate. *J Biol Chem* 271(47):29652–29658.
3. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226(1):497–509.

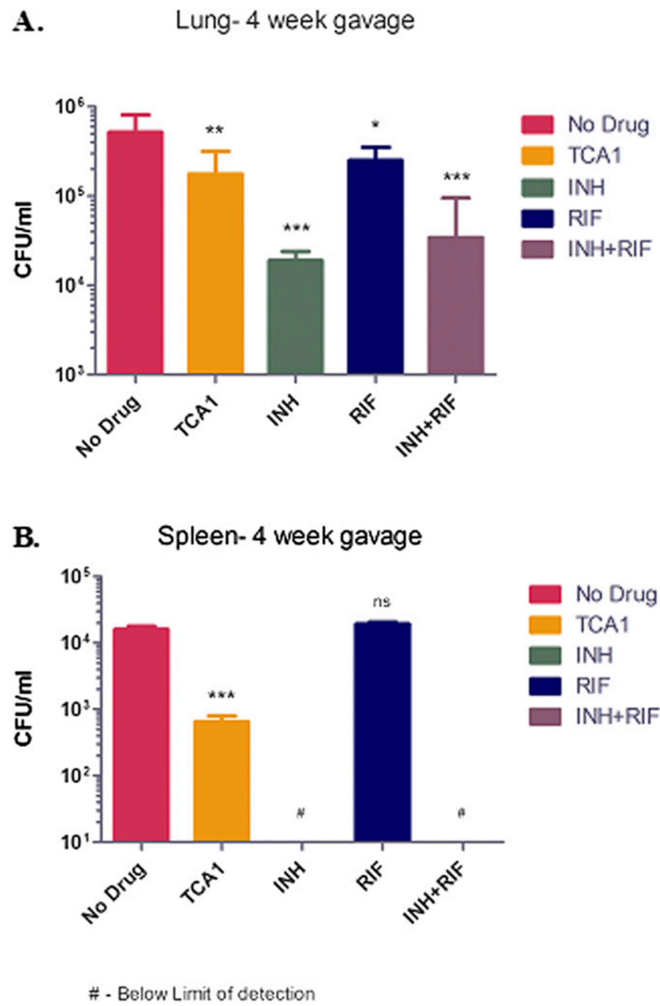

Fig. S1. In an acute tuberculosis infection mouse model (2 wk infection) followed by 4 wk of gavage (1 time/d and 5 d/wk), TCA1 showed bactericidal activity in both (*A*) lungs and (*B*) spleen. The doses of TCA1, isoniazid (INH), and rifampicin (RIF) are 40, 25, and 10 mg/kg, respectively. RIF and INH were administered in drinking water. *P* value less than 0.001.

Fig. S2. DprE1 is incubated with the drug of interest (different concentrations of TCA1) for 15 min. BTZ-BODIPY is , and the sample is incubated for 1 h at 37 °C. Samples are then analyzed by SDS/PAGE [Coomassie staining (*Upper*) and fluorescence scan (Lower)]. Lane 1: 9 μM DprE1, 20 μM FAD, 20 μM BTZ-BODIPY. Lanes 2–8: 9 μM DprE1, 20 μM FAD, 20 μM BTZ-BODIPY plus TCA1 (0, 50, 25, 12.5, 6.3, 3.1, and 1.6 μM).

Fig. S3. Chemical structure of molybdenum cofactor.

Fig. S4. In-gel fluorescence scanning. Probe-labeled MoeW was detected by click conjugation to a rohodmine-azide reporter tag followed by SDS/PAGE and in-gel fluorescence scanning. Lanes 1 and 2, 3 and 4, 5 and 6, and 7 and 8 are series of twofold dilutions of the *Escherichia coli* lysate. Lanes 1, 3, 5, and 7 are lysates of *E. coli* cells without isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. Lanes 2, 4, 6, and 8 are lysates of *E. coli* cells with IPTG induction. In lanes 4, 6, and 8, a band with the size of MoeW is present, whereas it is absent in noninduced samples (red arrows). The band is not very strong, because most of MoeW was found in inclusion body and not in soluble fraction when overexpressed in *E. coli*.

Table S1. Genotype of extensively drug-resistant tuberculosis strain

Strain no.	Strain name	Genotype	Drug resistance
$mc^2$8013	TF275	*inhA*[t1673432a] *katG*[S315T] *rpoB*[D435G] *embB*[M306V] *ethA*[t4327484c] *gidB*[ΔL50-P93) *pncA*[+gA152] *gyrA*[A90V] *rrs*[a1400g]	INH RIF EMB ETH SM pyrazinamide OXF KM CAP AM

Table S2. CYP activity and human cell cytotoxicity of TCA1

	IC_{50} (μM)
CYP inhibition	
CYP3A4	>10
Human cell cytotoxicity	
Huh7	>25
293T	>25
K562	>25
HepG2	>25
Vero	>100
hERG assay	>30

Table S3. Mean pharmacokinetic parameters of three mice after i.v. and p.o. administration

	TCA1		
	i.v. (10 mg/kg)	p.o. (20 mg/kg)	p.o. (50 mg/kg)
Area under the curve (h × nM)	13,268	4,724	12,851
Low clearance (mL/min per kilogram)	36.41	N/A	N/A
Steady-state volume of distribution (L/kg)	1.06	N/A	N/A
Cmax (nM)	20,195	2,122	5,653
Tmax (h)	0.03	0.5	0.5
$T_{1/2}$ (h)	0.73	1.8	1.83
MRT (h)	0.5	N/A	N/A
F (%)	N/A	46	19

p.o., per oral.

Table S4. Histopathology findings

	No drug					TCA1				
Diagnosis/severity	1	2	3	4	5	1	2	3	4	5
Lungs (*n* = 5)										
Pneumonia, granulomatous	0	1	1	2	1	0	0	0	0	0
Neutrophils within granulomatous foci	0	0	1	1	1	0	0	0	0	0
Infiltrate, perivascular, lymphohistiocytic	0	2	2	2	2	1	0	0	0	0
Acid fact bacteria in inflammatory foci	0	1	2	2	2	0	0	0	0	0
Spleen (*n* = 5)										
Histiocytic aggregates (granuloma)	3	3	2	2	2	0	0	0	0	0
Acid Fast organisms	3	2	1	2	1	0	0	0	0	0

Each slide had five tissue sections. Grading scale: 0, no finding; 1, minimal finding (for pneumonia = 0–10% lung involvement); 2, mild finding (for pneumonia = 11–30% lung involvement); 3, moderate finding. Note that a mild grading in the spleen is not the same as in the lung, because the lung generally has more acid-fast bacilli (AFB) than the spleen. A mild designation for AFB in the spleen is equivalent to a minimal designation in the lung.

Table S5. X-ray diffraction data and structure refinement

X-ray diffraction data	
Protein:ligand	DprE1:TCA1
Beamline	Diamond I02
Wavelength (nm)	0.9795
Space group	$P2_1$
Cell parameters a, b, c (Å), β (°)	78.2, 84.2, 81.1, 103.6
Molecules per asymmetric unit	2
Resolution (Å)	84.2–2.61
High-resolution shell (Å)	2.68–2.61
Rmerge (%)*	10.2 (99.2)
Observations: total/unique	148,069/31,083
$I/\sigma(I)$	13.1 (2.3)
Completeness (%)*	99.7 (99.0)
Multiplicity*	4.8 (4.9)
$CC_{1/2}$*†	0.997 (0.717)
Refinement	
Resolution range	62.5–2.6
Unique reflections	31,059
R_{free} (%)/R_{cryst} (%)	24.8/18.9
No. of nonhydrogen atoms	6,566
Protein + FAD, ligand, solvent	6,454, 50, 62
rmsd bonds (Å), angles (°)	0.008, 1.17
B factors	
Wilson ($Å^2$)	62.9
Average ($Å^2$)	55.5
Protein + FAD ($Å^2$)	55.2
Ligand ($Å^2$)	88.8
Solvent ($Å^2$)	57.5
rmsd B factors ($Å^2$)	7.3
Ramachandran plot‡	
Favored region	97.6
Allowed regions	2.4
Disallowed (no.)	0

$CC_{1/2}$, percentage of correlation between intensities from random half-datasets as defined in (1).

*Values for high-resolution shell are in parentheses.

†Pearson correlation coefficient between reflection half-sets as defined in Karplus and Diederichs (1).

‡Ramachandran plot statistics calculated using MolProbity (2).

1. Karplus PA, Diederichs K (2012) Linking crystallographic model and data quality. *Science* 336(6084):1030–1033.
2. Chen VB, et al. (2010) MolProbity: All-atom structure validation for macromolecular crystallography. *Acta Crystallogr D Biol Crystallogr* 66(Pt 1):12–21.